

Original Research Article

<http://dx.doi.org/10.20546/ijcmas.2016.510.114>

Isolation and Impact of Cellulose-Degrading Bacteria on Physico-chemical and Microbiological Properties of Plant Residues during the Aerobic Decomposition

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ABSTRACT

Five cellulose-decomposing *Bacillus* strains were isolated and the highly efficient two strains that were putatively identified as *Bacillus megaterium* and *Bacillus brevis* were used in the composting process. Plant residues were collected from the mown grass of King Abdulaziz University and composted for 60 days under aerobic conditions. Samples from the composting material were taken at 0, 12, 14, 33, 50 and 60 days and monitored for physical, chemical and microbiological analysis. The temperature of the compost pile rose to 44.0 °C after one day of composting and reached its peak of 55.0 °C after four days and lasted above 45.0 °C until 30 days. The pH value of the residues decreased during the first 14 days of the composting process to reach 5.0 and thereafter it increased to reach 8.6 at the end of this process. The organic matter and carbon content of plant residues gradually decreased during the composting process and the biodegradability coefficient of the composting process was 0.5. The C:N ratio was narrowed rapidly to reach 14.0 in the produced compost. The N, P and K contents of the residues increased with increasing the composting period to reach 3.0, 0.73 and 2.4%, respectively, in the resultant compost. Moreover, the number of total microbe count, cellulose-decomposing microorganisms, fungi and actinomycetes gradually decreased within the first 14 days of the composting process and then, they increased after 33 days except the actinomycetes, that continued to decrease up to 50 days of composting.

Keywords

Composting;
cellulose-
decomposing;
Bacillus; aerobic
decomposition;
Pile.

Article Info

Accepted:
25 September 2016
Available Online:
10 October 2016

Introduction

Composting means the biological decomposition of organic matter under controlled conditions. It indicates that the

process is managed or optimized to achieve desired objectives. One of these major objectives is to potentially decompose the

organic residues into a stable state and produce a material that may be applied for soil improvement or other beneficial uses (Epstein, 1997). Composting can be considered a microbial-based process which is an environmentally sound method that is adopted for reducing organic waste and to help in the production of organic fertilizers which are also so called organic conditioners (Gajdos, 1992). Biodegradable wastes could be composted to return the waste to the plant production cycle as a fertilizer or a soil improver. Therefore, the resulting compost is considered environmentally safe for use on soils that are in contact with plants, humans, and animals (Stratton *et al.*, 1995; Barker, 1997).

Many factors affect the composting process. Some of these factors play a major role in the process while others can influence its direction or extent. These factors, in their turn, strongly affect the structure and diversity of the microbial community, microbial activity and physical and chemical characteristics of the substrate (Miller, 1993). The major factors that influence the decomposition of organic matter by microorganisms are oxygen and moisture. Temperature is also an important factor in the composting process; however, it is the result of the microbial activity. Other important factors that could limit the composting process include nutrients and pH value, as well as the particle-size and carbon to nitrogen (C:N) ratio of the organic materials. The nutrients, especially carbon and nitrogen, are needed for cell synthesis. The aerobic windrow composting is the least technologically advanced and the oldest form of controlled composting (Komilis and Ham, 2004). Researches that are applied on different composting systems highlighted on the open-turned, aerobic windrow composting as the lowest cost system in both set up and operation (Xi *et al.*, 2005).

The compost nutrient value varies widely, depending upon the nature of the composted feedstock. If the initial material contains manure, it will be richer in nitrogen and other nutrients. According to W. S. U. (2004), most of the finished composts contained organic matter of 25 to 50 %, carbon of 8 to 50 %, nitrogen of 0.4 to 3.5 %, phosphorus as P₂O₅ of 0.3 to 3.5, potassium (as K₂O) of 0.5 to 1.8 %, and calcium (as Ca O) of 1.5 to 7.0 %. This study aims to isolate cellulolytic bacteria, follow up and understand the changes in the physico-chemical and microbiological characteristics of plant residues during their aerobic decomposition.

Materials and Methods

Isolation of cellulose-decomposing microorganisms

Samples were collected from various places i.e., a sheep dung soil, a horse waste of King Abdulaziz University (KAU) Equestrian Club, a mature compost, and a rhizosphere soil of *Ixora* sp. plants. A serial dilution technique was used for the isolation of cellulolytic bacteria. Thus ten grams of each sample were added to 90 ml sterilized distilled water in 250 ml dilution bottle to give 10⁻¹ dilution and thoroughly mixed, then further serial dilutions were made up to 10⁻⁶. Aliquots of one ml from 10⁻⁴, 10⁻⁵ and 10⁻⁶ dilutions were transformed to sterile Petri dishes. A carboxymethyl cellulose (CMC) agar medium was used for the isolation and subsequent cultivation and it was constituted of 5 g carboxymethyl cellulose (CMC), 5.0 g of Peptone, 5.0 g of NaCl, 3.0 g of beef extract, 18.0 g of agar at pH 7.00 and 1000 ml of distilled water (Sakthivel *et al.*, 2010). The melted (CMC) medium was poured and well mixed with the added inoculums. Then, the inoculated Petri dishes were incubated at 30°C for 48

hr. Bacterial colonies were purified by streaking on nutrient agar plates and incubated at 30°C for 24 hr. The pure isolated colonies were maintained on the CMC agar slants at 4°C for further analysis.

Screening the isolated bacteria for cellulase activity

The disc diffusion method was applied for the relative activity determination of cellulase production among various isolates. According to Sakthivel *et al.* (2010), the agar plates were prepared with 0.5% CMC and 2.0% agar. Filter paper discs of one cm diameter were saturated with either the entire culture or its supernatant of the respective isolate and then, transferred onto the CMC medium under sterile conditions. The control filter paper discs were saturated with distilled water. The plates were incubated at 30°C for 48 hr. After the incubation period, the medium was flooded with a congo red dye solution (0.1% w/v). The plates were further treated by flooding with 1M NaCl for 15 min. The hydrolytic zone was measured using a ruler and recorded.

Identification of the isolated microorganisms

The obtained isolates were identified using the criteria of Bergey's manual of determinative bacteriology (Bergey *et al.*, 1994). The morphological and biochemical tests of colony and cell morphology, endospore forming, Gram's stain, carbohydrate fermentation (glucose, mannitol, arabinose), citrate utilization, catalase, gelatinase production, Voges-Proskauer test, methyl red test, hydrolysis of starch and growth in 6.5% NaCl, were applied.

Inoculum preparation

Batch cultures of the selected strains of

cellulase decomposers were separately prepared and thoroughly mixed before their application to the pile of plant residues. In addition, a diluted carbohydrate solution was prepared from date molasses (1:20 w/v) to be applied to the pile of the plant residues as an easy nutrient source for the applied cellulase decomposing microbial strains.

Pile formation

Plant residues (grass clippings) were collected from the gardens of KAU and stockpiled in a safe place, until a sufficient quantity was collected. A windrow of the grass residues of 3.5 m length, 2.0 m width and 1.0 m height was made of different layers. Then, each layer was moistened and inoculated with the respective selected cellulose decomposers strains to finally get a moisture content 60-70% of WHC. Eventually, the windrow was covered with a plastic sheet to prevent the fast drying of the grass residues. The windrow was overturned and moistened (according to the need) after 14, 33, 50 and 60 days according to elevation of the compost temperature.

Compost sampling

The sampling was performed according to Mahdy *et al.* (2012). Combinations of three samples were taken from the top, middle and bottom of the whole pile profile after each turning at 0, 14, 33, 50, and 60 days. All collected three samples were thoroughly mixed in plastic bags in order to make one kilogram sample.

Sample analyses

Samples were collected from the compost during the different decomposition stages for the determination of some physico-chemical and microbiological parameters. The chemical analyses were performed on

dry samples while pH and the microbiological analyses were done on moist ones. Three replicates were used for the physico-chemical analyses except temperature where five replicates were applied. Five replicates were also taken for microbiological analyses.

Physico-chemical parameters

Temperature

Compost temperature was measured daily in the morning by a thermometer according to Bhoyar *et al.* (1979). A thermometer was inserted inside the compost pile in random different places and the reading was recorded after stabilization. The daily ambient temperature at that time was also determined.

Moisture content

The moisture content of the compost samples were determined using an electric oven at 105°C for 24 hr. according to Mooijman and Lustenhouwer (1978). The samples were cooled in a desiccator and the weight was recorded. The weight loss was expressed as a percentage with reference to the initial dry weight.

Compost pH

The pH of compost samples was measured in a 1:5 compost suspension of compost to distilled water ratio (w/v) (Hue and Liu, 1995).

Organic matter Determination

The organic matter content was determined by burning the compost samples at 550°C for three hours to be ash in a muffle-furnace. Then the organic matter was calculated as a percentage as follows (Tiquia and Tam, 1998):

Organic matter % = [(Dry weight – Ash weight) / Dry weight] × 100

Carbon content

The organic matter percentage contains 58-60 % of organic carbon (Nelson and Sommers, 1996). The organic carbon content of the compost samples was calculated as percentage as follows: Carbon content % = Organic matter % ÷ 1.7.

Nitrogen content

The nitrogen content of the compost samples was determined using micro- Kjeldahl method (Kjeldahl Digestion: Tector, Digestion system 40 1016, Germany and Kjeldahl Distillation: Gerhardt, Vapodest-Germany) according to Bremner and Mulvaney (1982).

C:N ratio estimation

The ratio between nitrogen to carbon content of the compost samples was calculated as follows:

C:N ratio = Carbon content % ÷ Nitrogen content %

Macronutrient and heavy metal contents

The estimation of some macronutrients (K and P) content and some heavy metals (Pb, Zn and Cd) was determined using the inductively coupled plasma optical emission spectrometry 4300 dual view (ICP- OES 4300 DV).

Microbiological parameters

The total aerobic mesophilic microbes were determined using the dilution plate count technique on nutrient agar according to Hassen *et al.* (2001). The number of cellulolytic aerobic bacteria were estimated

by plating the appropriate dilutions of samples on the CMC agar medium based on clear zones formation after staining with Congo red (0.1% w/v) for 15 min and then, flooding with 1M NaCl for 15 min. The number of viable fungi was measured by plating appropriately diluted suspensions onto rose bengale agar according to Smith and Dawson (1944). A starch-Nitrate agar medium was used for actinomycetes according to Atta *et al.* (2011).

Results and Discussion

Screening the cellulytic activity of isolated bacteria

Based on the observations of the diameters of the clear zone surrounding the bacterial colonies and distinct colony morphologies, 5 of 12 cellulytic bacteria were selected for further characterization. Two strains (Sh1 and Sh2) were isolated from the sheep dung and two stain (Rh1, Rh2) were from the rhizosphere and one strain (C) was from the mature compost. The pure isolates were kept on CMC agar slants in the fridge at 4.0°C for one week.

The cellulytic activity of bacterial strains (Sh1, Sh2, Rh1, Rh2 and C) is shown in Fig.1. The Sh2 and C strains exhibited higher clear zones on CMC agar plate than the other strains at different incubation periods (Table 1). The cellulytic activities of all strains increased with the incubation period. The highest cellulytic activity for all tested strains was found at 120 hr. incubation period while the least one was observed at 24 hr. incubation period.

Identification of isolated strains

Depending on the cellulytic activity the five, isolated bacterial strains were selected for identification. The morphological and physiological characteristics of the isolated strains are presented in Tables 2 and 3,

respectively. Therefore, these bacterial strains were putatively identified as Sh1 strain (*Bacillus popilliae*), Sh2 strain (*Bacillus megaterium*), Rh1 strain (*Bacillus badius* 1), Rh2 strain (*Bacillus badius* 2) and C strain (*Bacillus brevis*).

Physico-chemical characteristics of plant residues

Plant residue samples were taken and analyzed during the composting process at 0, 14, 33, 50 and 60 day to investigate the changes in their properties. The physico-chemical properties of these samples are shown in Table (4).

Pile temperature

The temperature of the plant residue pile increased to 44.0 °C after one day of the composting and reached its peak of 55.0 °C after four days and lasted above 45.0 °C until 30 days (Table 4). After 30 days the temperature slowly decreased to reach 38.0 °C up to 60 days of the composting process. The temperature increased after each turning on the 14th and 33rd days. The ambient temperature was between 33.0 and 41.0 °C during the composting period. This result is in an accordance with those of other investigators (Tiquia *et al.*, 2002; Liu *et al.*, 2011; Kutsanedzie *et al.*, 2012). The rapid increasing temperature may be attributed to introducing more active microorganisms as a result of their inoculation which they increased the rate of organic matter decomposition (Hanajima *et al.*, 2006).

Moisture content

The moisture content of the composted plant residues gradually increased from 71.0 % to 73.0 % during the first 14 days and thereafter it decreased to reach 25.0 % up to 60 days of composting (Table 4). After 50 days, the composting material were allowed

to be dried naturally. An optimum moisture content of the compost is important for the microbial decomposition of organic wastes. In this study the moisture content was maintained above 50% during the composting period as it is recommended by Gazi *et al.* (2007).

Compost pH

The initial pH value of the composting material was 8.0 and then, it rapidly decreased during the first 14 days of composting to reach 5.0. Thereafter, it increased to reach pH 8.6 after 60 days (Table 4). The initial decrease in pH during the first period of composting is expected because of the formed acids during the metabolism of readily available carbohydrates (Beck-Friis *et al.*, 2003; Gautam *et al.*, 2010). However, the pH rises after that above 7.0 because the formed acids are consumed by microorganisms and ammonia is produced from protein degradation. At the end of composting process, the compost was slightly alkaline. The same trend was recorded by other investigators (Sundberg and Jonsson, 2005; Gazi *et al.*, 2007; Abu-Zahra *et al.*, 2014).

Organic matter

The organic matter content of the composting residues was slowly reduced from 81.0 % to 79.5 % within 33 days of the composting period. After 33 days the organic matter rapidly decreased to reach 69.0 % up to 60 days of the composting (Table 4). Also, organic carbon content gradually decreased from 49.0 % at the zero time to 41.0 % at the end of the composting process. The reduction in the total organic matter during the composting process is mostly due to the degradation of the easily degradable compounds such as proteins, cellulose and hemi-cellulose, which they are utilized by microorganisms as carbon and

nitrogen sources (Barrington *et al.*, 2002). Also, enriching the fresh wastes with cellulose decomposing microorganisms leads to a higher rate of organic matter oxidation (Saber *et al.*, 2011).

C:N ratio

The C:N ratio of the composting material started to narrow after 14 days of composting period and continued to narrow to reach 14.0 up to 60 days (Table 4). The C:N ratio showed a downward trend during the composting process. These findings were supported by Sarkar *et al.* (2010) and Jusoh *et al.* (2013). The current results indicated that the C:N ratio decreased from 41.0 to 14.0 during the composting period due to the loss of carbon content and an increase in the nitrogen content per unit material.

Macronutrients and heavy metals

The total nitrogen content of the composting material rapidly increased during the composting process from 1.2 % at the zero time to 3.0 % after 60 days (Table 4). The obtained results also showed that potassium (K) and phosphorus (P) contents increased during the composting period up to 60 days. The potassium content increased from 0.76 % at the initial time to 2.4 % in the final product while the phosphorus increased from 0.26 % to 0.73 % during this period. According to Zhu (2007), the increase in the total nitrogen may be related to microorganism's nitrification process. In addition, nitrogen content can also be increased by the activities of nitrogen-fixing bacteria at the end of the composting process (Bishop and Godfrey, 1983). Regarding the P and K contents, they showed an increasing trend during the composting process. These results are in accordance with those of other investigators who confirm the same trend for P and K in

the compost (Georgacakis *et al.*, 1996; Tai and He, 2007; Selim *et al.*, 2012; Jusoh *et al.*, 2013). The P content reached 0.73 % and K content was 2.4 % in the final product. It is worth mentioning that the normal range of P in the compost is between 0.4 to 1.1 %, while for K it ranges from 0.6 - 1.7 % (Bord na Mona, 2003). This suggests that the P content of the final

compost is within the normal range but the K content is greater than the upper threshold level of the normal range.

Lead (Pb) and zinc (Zn) contents of the composting materials showed an increasing trend during the composting period up to 60 days.

Table.1 The clear zone diameter (cm) of the isolated strains in the carboxymethyl cellulose (CMC) agar medium at different incubation periods

Bacterial strain	Incubation period (hr.)				
	24	48	72	96	120
Sh1	0.5	1.7	2.1	2.2	3.0
Sh2	0.9	2.2	2.8	3.7	4.4
Rh1	0.2	0.4	0.7	0.85	1.2
Rh2	0.3	0.6	1.2	1.2	1.6
C	1.4	2.1	3.0	3.4	4.1

Table.2 The colony and cell morphology of the isolated bacterial strains.

Colony and Cell Morphology		Bacteria strain				
		Sh1	Sh2	Rh1	Rh2	C
Colony morphology	Form	Circular	Irregular	Irregular	Irregular	Irregular
	Elevation	Flat	Umbonate	Flat	Flat	Umbonate
	Margin	Entire	Curled	Undulate	Undulate	Curled
	Color	Creamy or Buff	Creamy or Buff	White	White	Creamy or Buff
Growth in nutrient broth		Surface	Surface	Spread	Spread	Surface
Cell morphology		Single rods	Single rods	single rods & in short chains	rods in long chains	single rods & in short chain
Endospore formation		+	+	+	+	+
Spore position		Central and Swelling	Central	Central	Sub-terminal	Central and Swelling

Table.3 The physiological test for the identification of the isolated strains

Test	Bacterial strain					
	Sh1	Sh2	Rh1	Rh2	C	
Gram's stain	+ ve	+ ve	+ ve	+ ve	+ ve	
Starch hydrolysis	- ve	+ ve	+ ve	+ ve	+ve	
VP test	- ve	- ve	- ve	- ve	- ve	
Catalase	- ve	- ve	- ve	- ve	- ve	
Citrate utilization	- ve	+ ve	- ve	- ve	- ve	
Gelatinase	- ve	+ ve	- ve	- ve	- ve	
Methyl red test	-ve	- ve	- ve	+ ve	- ve	
Growth in 6.5% NaCl	- ve	+ ve	+ ve	- ve	+ ve	
Sugar Fermentation	Glucose	+ ve	+ ve	+ ve	+ ve	+ ve
	Manitol	+ ve	- ve	- ve	- ve	+ ve
	Arabinose	- ve	- ve	- ve	- ve	- ve

- ve = Negative and + ve = Positive.

Table.4 Some physico-chemical parameters of plant residues during composting process

Parameter	Raw Material	Composting period (Day)				
		0	14	33	50	60
Temperature (°C)	-	41.0	46.0	45.0	43.0	38.0
Moisture content (%)	63.0	71.0	73.0	67.5	55.0	25.0
pH	7.00	8.00	5.00	6.00	8.40	8.60
Organic matter (%)	78.0	81.0	80.0	79.5	69.0	69.0
Nitrogen content (N %)	1.30	1.20	2.50	2.00	3.00	3.00
Carbon content (C%)	47.0	49.0	48.0	48.0	41.0	41.0
C:N ratio	36.0	41.0	19.0	20.0	14.0	14.0
Potassium content (K %)	0.88	0.76	1.55	2.00	2.35	2.40
Phosphors content (P %)	0.32	0.26	0.48	0.49	0.60	0.73
Lead (Pb ppm)	2.00	3.00	4.00	8.00	9.00	9.00
Zinc (Zn ppm)	31.0	30.0	50.0	80.0	100	110
Cadmium (Cd ppm)	< 1	< 1	< 1	< 1	< 1	< 1

Fig.1 The cellulytic activity of the selected strains on the carboxymethyl cellulose (CMC) agar medium after 120 hr. of incubation at 30° C.

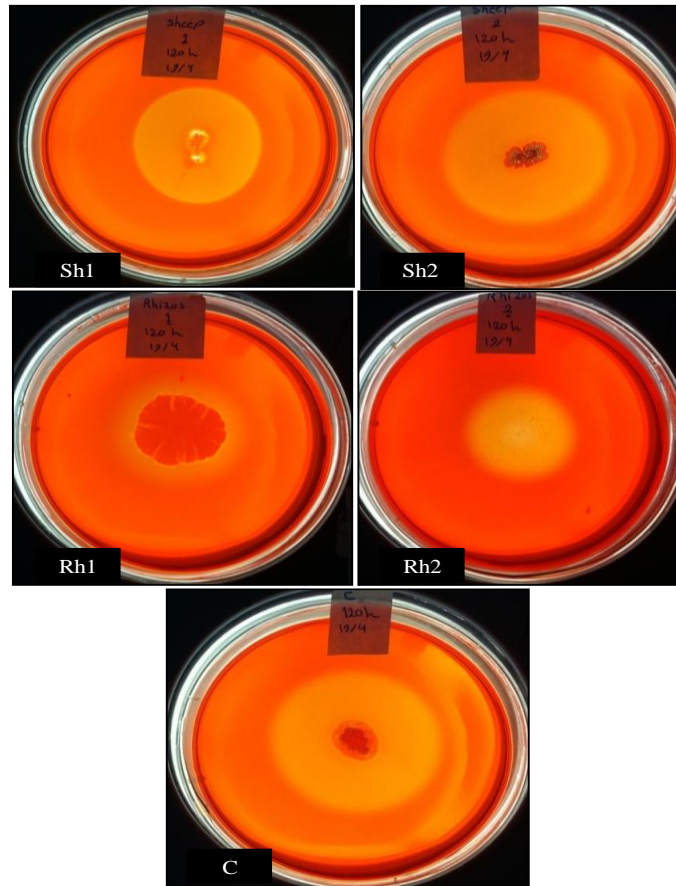
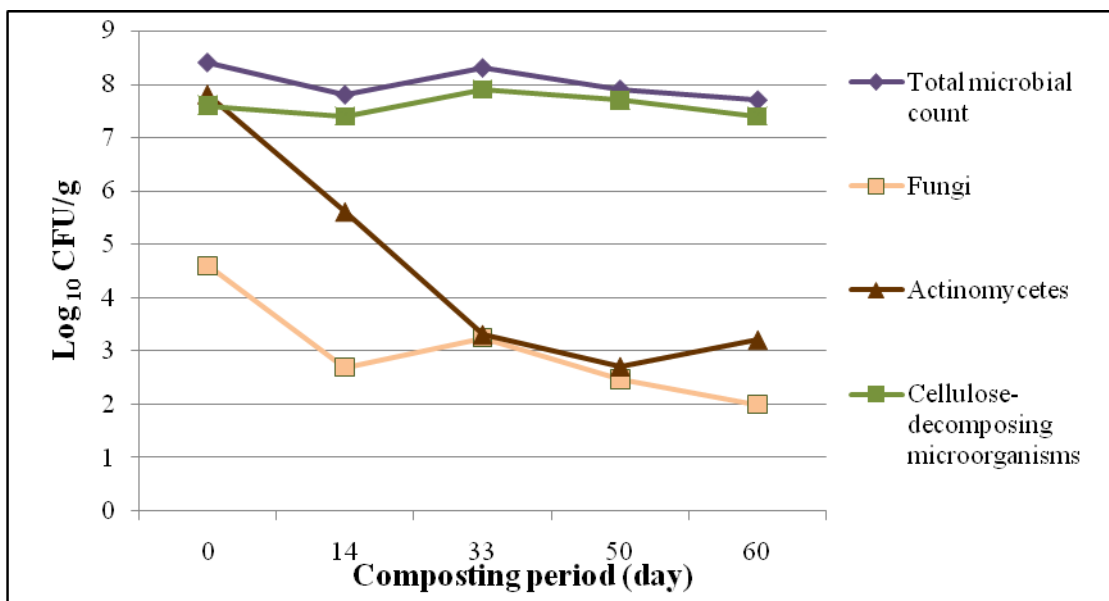


Fig.2 Microbial population count variations during composting process.



The Pb-content increased from 3.0 ppm at the initial time to 9.0 ppm at the end of the composting process while Zn increased from 30.0 ppm to 110 ppm. However, cadmium (Cd) content recorded the least amount among the determined heavy metals (<1.0 ppm). This observation is in an agreement with Paré *et al.* (1999) who obtained similar results during composting where nutrient and heavy metals accumulation significantly increased, indicating the maturity of the product. In the present study, Zn, Pb and Cd concentrations in the produced compost were 119 ppm, 9 ppm and >1 ppm respectively. The upper limit value that is recommended by British Standard Institute (2011) is 400 ppm for Zn, 200 ppm for Pb and 1.5 ppm for Cd. It is interesting to note that the concentrations of these metals at the end of the composting process were below the limits proposed by the regulatory agency.

Microbiological analysis characteristics

The fluctuations in microbial counts during the various stages of composting process were shown in Fig. 2. Regarding the total microbial count, the highest numbers were recorded in the raw material and at the initial time of composting i.e., 2.8×10^8 CFU/g and 2.5×10^8 CFU/g respectively while the least number was found at the end of composting process to record 5.3×10^7 CFU/g. On the other hand the minimum number of cellulose-decomposing microorganisms (2.0×10^6 CFU/g) was recorded at raw material while their maximum number (7.2×10^7 CFU/g) was recorded after 33 days of composting process and their numbers slightly declined. (Fig. 2). For actinomycetes, it was found that the highest number was at the zero time and then gradually decreased up to 50 days. On the other hand, the minimum recorded numbers of microorganisms in the compost

were found for fungi that ranged from 1.0×10^2 to 7.5×10^4 CFU/g. Their highest number was found in the raw material and the lowest one was after 60 days of the composting process. The same trend was recorded by other investigators confirming this decline could be attributed to the fact that during the curing phase the cellulose may become inaccessible to the enzymatic attack because of low water content or association with protective substances such as lignin (Ryckeboer *et al.*, 2003 Gazi *et al.*, 2007; El-Akshar *et al.*, 2012).

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How to cite this article:

Ebtihal Abdullah Motwali, Hashem Mahmoud Mohamed, Abu-Bakr Mahmoud Gomaa and Fahad Abdul Rahman Al-Fassi. 2016. Isolation and Impact of Cellulose-Degrading Bacteria on Physico-chemical and Microbiological Properties of Plant Residues during the Aerobic Decomposition. *Int.J.Curr.Microbiol.App.Sci.* 5(10): 1084-1096.
doi: <http://dx.doi.org/10.20546/ijcmas.2016.510.114>